Heterodimers and family-B GPCRs: RAMPs, CGRP and adrenomedullin

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Abstract

RAMPs (receptor activity-modifying proteins) are single-pass transmembrane proteins that associate with certain family-B GPCRs (G-protein-coupled receptors). Specifically for the CT (calcitonin) receptor-like receptor and the CT receptor, this results in profound changes in ligand binding and receptor pharmacology, allowing the generation of six distinct receptors with preferences for CGRP (CT gene-related peptide), adrenomedullin, amylin and CT. There are three RAMPs: RAMP1–RAMP3. The N-terminus appears to be the main determinant of receptor pharmacology, whereas the transmembrane domain contributes to association of the RAMP with the GPCR. The N-terminus of all members of the RAMP family probably contains two disulphide bonds; a potential third disulphide is found in RAMP1 and RAMP3. The N-terminus appears to be in close proximity to the ligand and plays a key role in its binding, either directly or indirectly. BIBN4096BS, a CGRP antagonist, targets RAMP1 and this gives the compound very high selectivity for the human CGRP1 receptor.

RAMPs (receptor activity-modifying proteins) and the CT (calcitonin) family of peptides

GPCRs (G-protein-coupled receptors) can be divided into several families. Family B includes receptors for 30–50-amino-acid peptides. The CT family of peptides are typical of the endogenous ligands for these receptors. This family includes CT, CGRP (CT gene-related peptide), AM (adrenomedullin) and AMY (amylin). Although these show only limited sequence homology, they share several features of secondary structure. Biological activity is conferred by the N-terminus; at its core, it consists of a five- or six-residue disulphide-bonded peptide. The remainder of the molecule, approx. 30 amino acids, is required for binding [1]. There is extensive cross-reactivity between the receptors for these peptides. It is now clear that the reason for this is that the receptors are heterodimers, sharing common components. Two seven-transmembrane proteins, the CT receptor and CL (CT receptor-like receptor), can combine with three RAMPs to generate six pharmacologically distinct receptors (Table 1). With the inclusion of various splice variants of the CT receptor in this scheme, even greater diversity can be accommodated.

RAMP function

RAMPs were first discovered in 1998 by McLatchie et al. [2] as proteins that could confer function to the then orphan receptor, CL. RAMP1 produced a CGRP receptor; RAMP2 gave an AM receptor, and RAMP3 gave an additional AM receptor but with appreciable affinity for CGRP. It should be emphasized that, in the absence of RAMPs, CL will not interact with any known ligand. Unlike CL, the CT receptor will bind CT with high affinity in the absence of any other protein [3–6]. However, RAMPs can also associate with it to produce receptors with enhanced affinity for AMY; the RAMP1 and RAMP3 heterodimers also have significant affinity for CGRP (although the precise phenotype depends on the cell line used for expression, the CT receptor splice variant and the G-proteins present; [7–10]). Most pharmacological characterization of RAMPs has been performed with RAMP1 and RAMP2, investigating their interaction with CL (see [1] for a summary). However, whereas RAMP3 is not particularly abundant in rats, this is not the case in humans [2], where CL–RAMP3 and CT–RAMP3 complexes are probably much more abundant. The recent literature on CGRP pharmacology is complex, with much discussion of a ‘CGRP2’ receptor subtype for which there is currently no molecular correlate [1]. This may reflect CGRP action on heterodimers such as CL–RAMP3 and CT–RAMP1, which have significant affinity for CGRP, while they are formally considered as receptors for other peptides [1].

Although the most significant effects of RAMPs have been seen with the CL and CT receptors, they can interact with other family-B GPCRs [11] (Table 2). In some cases, there are no observable effects on receptor pharmacology (although subtle changes, perhaps in coupling, cannot be ruled out). However, the VPAC1–RAMP2 complex shows enhanced phosphoinositide hydrolysis, with no change in cAMP production, probably reflecting improved accessibility to Gq or phospholipase C [11].

Key words: adrenomedullin, calcitonin gene-related peptide (CGRP), calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1), RAMP2, RAMP3.

Abbreviations used: AM, adrenomedullin; AMY, amylin; CT, calcitonin; CGRP, CT gene-related peptide; CL, CT receptor-like receptor; GPCR, G-protein-coupled receptor; RAMP, receptor activity-modifying protein.

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Table 1 | Relative potencies of ligands at the CL and CT receptor complexes

Based on Tables 3 and 4 in [1] and references cited therein; h, human; r, rat.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Potency order</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCL</td>
<td>No binding</td>
</tr>
<tr>
<td>hCL/hRAMP1</td>
<td>hAM &gt; hCGRP</td>
</tr>
<tr>
<td>hCL/hRAMP2</td>
<td>hAM &gt; hCGRP</td>
</tr>
<tr>
<td>hCL/hRAMP3</td>
<td>hAM &gt; hCGRP</td>
</tr>
<tr>
<td>hCT(a)</td>
<td>hCT &gt; rAMY &gt; hAM</td>
</tr>
<tr>
<td>hCT(a)/hRAMP1</td>
<td>hAMY &gt; hAM</td>
</tr>
<tr>
<td>hCT(a)/hRAMP2</td>
<td>rAMY &gt; hAM, hCT</td>
</tr>
<tr>
<td>hCT(a)/hRAMP3</td>
<td>rAMY = hAMY = hAMY</td>
</tr>
</tbody>
</table>

Table 2 | Interaction of RAMPs with family-B GPCRs

Based on [11,29]. No interactions were seen with VPAC2 (vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide 2), GLP (glucagon-like peptide)-1, GLP-2 and GHRH (growth-hormone-releasing hormone) receptors.

<table>
<thead>
<tr>
<th>RAMP</th>
<th>CL</th>
<th>CT receptor</th>
<th>VPAC1</th>
<th>PTH1</th>
<th>PTH2</th>
<th>Glucagon receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAMP1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>RAMP2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RAMP3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 1 | Alignment of human RAMPs

... identical in all the RAMPs; N, potential glycosylation site; MAS..., signal peptide; WAM..., domain implicated in AM binding; FFL..., domain implicated in CGRP binding.

RAMP1 has no glycosylation sites. It is unable to reach the cell surface by itself and it accumulates intracellularly. It requires heterodimerization with CL for cell-surface expression, but can be induced to move to the cell surface by the introduction of glycosylation sites ([16], but see below for further discussion on this issue). RAMP2 and RAMP3 have multiple glycosylation sites. There appears to be variable glycosylation of RAMP3 using between two and four of its potential sites [12]. Removal of all these sites impairs but does not abolish AM binding and receptor activation, showing that they are not essential for receptor activation. Interestingly, if all the disulphide bonds in RAMP3 are abolished by cysteine mutation, all the glycosylation sites will be fully utilized. This implies that enzymic access to glycosylation sites in the correctly folded protein is restricted; they are all next to cysteine residues. It is not clear how far these results can be extrapolated to RAMP2 since only one of the glycosylation sites found in RAMP3 is also conserved in human RAMP2.

Studies with chimaeric RAMPs have shown that the N-terminus of RAMP1 is the key determinant for the expression of CGRP binding [17,18]; this interacts with the N-terminus of CL. The isolated N-terminus of RAMP1 by itself is sufficient to confer CGRP binding on CL, albeit with a much reduced affinity [19]. Broadly similar conclusions have been drawn from studies on RAMP interactions with the CT receptor, where the N-terminus of the RAMP is the chief determinant of receptor pharmacology [9]. The transmembrane helix appears to be important for association with CL and the CT receptor. The residues QSKRT immediately distal to the transmembrane domain of RAMP1 serve to retain the protein in the endoplasmic reticulum; if these are deleted, even the non-glycosylated protein can reach the cell surface [13]. The remaining five residues of the C-terminus have no known function but it cannot be ruled out that they play a role in receptor localization or interaction with various effectors.

RAMP structure

The RAMP family consists of three proteins, each member being approx. 150 amino acids long (Figure 1). Each RAMP has a short intracellular C-terminus of approx. 10 amino acids followed by a single transmembrane region of approx. 20 amino acids. The remainder of the protein consists of the extracellular N-terminus with a signal peptide. The extracellular domain of RAMP2 is longer than those of RAMP1 and RAMP3, partly due to a 12-amino-acid insert towards its N-terminus. The extracellular domain has four cysteine residues conserved between all members of the RAMP family and these may be expected to form two disulphide bonds, although the arrangement is unknown. Disruption of these bonds abolishes or impairs function in all the RAMPs [12–14]. RAMP1 and RAMP3 have an additional pair of cysteine residues; these may form an extra disulphide bond but mutation of either residue does not impair CGRP binding in CL–RAMP1 heterodimers [13]. Thus, even if they form a disulphide bridge, it is not essential for function. RAMPs do not form disulphide bonds with CL; however, at least RAMP1 can exist as a disulphide-bonded homodimer in the endoplasmic reticulum before transport to the cell surface. Association with CL leads to intramolecular disulphide bond formation and cell-surface transport [15].

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Mechanism of action of RAMPs

The mechanism of action of RAMPs has undergone close examination. Initially, it was suggested that they altered the
glycosylation state of the receptor, but this now seems to be an artifact [20,21].

Normally, CL and the RAMPs associate in the Golgi and are co-trafficked to the cell surface [2]. Co-immunoprecipitation studies have shown that they are stable complexes at the cell surface and that they maintain their association even after internalization [21–23]. The RAMPs interact with CL to produce an appropriate conformation of the ligand-binding pocket. CGRP and AM can be cross-linked to RAMP1 or RAMP2 in addition to CL [20,21], indicating that the ligands are in close proximity to both proteins. Both CGRP and AM have been reported to label 17 kDa bands in cross-linking experiments conducted on a variety of tissues [24,25]; this is the molecular mass that would be expected for RAMPs.

Little detail is available as to how RAMPs and the N-terminal domains of the target GPCR interact to create ligand-binding sites. Although the radioligand-cross-linking studies are quite informative as they suggest that parts of the RAMP are in close proximity to the ligand, it is not known if any specific contacts are made, or whether the RAMPs primarily act by inducing a new conformation of the N-terminus of GPCR. Some domains involved in ligand binding have been identified from studies on chimaeric RAMPs. It has been proposed that residues 86–92 of human RAMP2 and residues 59–65 of human RAMP3 are needed for AM binding [26,27]; these occur in homologous parts of the two proteins. For RAMP1, residues 91–103 are needed for CGRP binding, whereas residues 78–90 appear to be involved in AM binding [28]. Both of these sections of the protein are proximal to the domains identified in RAMP2 and RAMP3 for AM binding. Throughout these studies, no single residue could be identified as being critical for the production of CGRP or AM pharmacology, although deletion of certain residues markedly altered receptor expression. Although detailed binding analysis of the mutants is still awaited, it seems that the epitopes act, at least in part, to bring the RAMPs and CL together, probably acting in concert with the transmembrane domain. Interestingly, in RAMP1, mutation of Leu-94 to an alanine residue increases CGRP binding; this has been interpreted as relieving the steric hindrance caused by the leucine side chain, allowing a more efficient formation of CL–RAMP1 heterodimers [29]. Further analysis of the RAMPs is necessary to identify how they modulate GPCR phenotype.

Examination of the sequences of RAMPs suggests some further clues as to how they might function. The extra putative disulphide in RAMP1 and RAMP3 may stabilize a loop that is important for CGRP binding; it connects the N-terminus of the molecules close to the start of the domain needed for CGRP binding in RAMP1. The consensus sequences at either end of this loop (NH2-CN/QE…GTV…DLGF) are very different from the RAMP2 sequence (…GTV…DLGF). The N-terminal extension of RAMP2 may further contribute to selectivity by preventing the formation of this loop. We speculate that the disulphide loop works with other sequences within the middle section of RAMP1 to dock CGRP, either directly or with a part of CL. For RAMP1, this may allow the N-terminus of CGRP to make high-affinity contacts with the transmembrane/juxtamembrane portion of CL leading to activation. For RAMP3, the docking appears less favourable; hence, the N-terminus of CGRP would not be capable of making such favourable contacts.

The RAMP extracellular domains are expected to fold independent of the membrane and intracellular domains. Accordingly, we have expressed the extracellular domain of RAMP1 as a glutathione S-transferase-fusion protein. Using the commercially available expression vector pGEX-6p-1, the protein has been produced in Escherichia coli, where it accumulates in inclusion bodies. We have successfully developed a method to refold the protein. This involves solubilization in 6 M guanidinium chloride in the presence of a reducing agent, dilution into refolding buffer with an oxidizing agent to ensure correct formation of disulphide bonds and then dialysis against PBS. After concentration against PEG 8000, analysis by SDS showed a band with the predicted molecular mass of 40 kDa for the fusion construct. This can be concentrated to >5 mg/ml without precipitation; this high solubility is powerful circumstantial evidence that it is in a stable state. Thus we have produced the extracellular domain of RAMP1 as a glutathione S-transferase-fusion protein in a form that is soluble at high concentrations. Further work is needed to cleave this and characterize the resulting protein. However, if this is monomeric and retains high solubility, this would allow us to initiate structural characterization.

**RAMPs as drug targets**

Principally, RAMPs should be targets for novel therapeutic agents. A novel CGRP antagonist appears to owe its selectivity to an interaction with RAMP1. BIBN4096BS is a non-peptide antagonist with pKd > 10 at human CGRP receptors (i.e. CL–RAMP1 heterodimers), but it is much less potent at non-primate receptors or at AM receptors (CL–RAMP2 and CL–RAMP3) [30]. In human RAMP1, substituting the tryptophan residue at position 74 with the lysine (found in rat RAMP1) decreases the affinity of the antagonist to that found in rat CL–RAMP1 heterodimers [31]. Although RAMP1 by itself will not bind BIBN4096BS, showing that CL is required (either directly or indirectly), the antagonist can be considered to be primarily targeted against RAMP1.

This work was supported by the Wellcome Trust, the BBSRC and the British Heart Foundation (PG/03/079/17278).

**References**


Received 15 June 2004